Moisture Sensitivity of Cotton Pollen: An Emasculation Tool for Hybrid Production

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ABSTRACT

Production of cotton (*Gossypium hirsutum* L.) hybrids is commonly preceded by removing anthers from recipient flowers or interrupting the functioning of the anthers before dehiscence. This study describes the development of an emasculation protocol that did not require removal of the anthers, did not result in female sterility, and maintained fruit retention. Cotton flowers were saturated with water at different times throughout the day. Water sprayed into the flower after the pollen dehisced resulted in the osmotic disruption of the pollen grains and prevented self-pollination of the cotton flowers. Morphological analysis of isolated pollen grains before and after water treatment showed the exudation of pollen cytoplasm into the surrounding water medium within seconds of the water treatment. The water treatment resulted in the loss of fruiting bodies. Hand pollination of water-emasculated flowers produced seed numbers equivalent to self-pollinated controls. To quantify the level of self-pollination following water emasculation, 'Gregg 65' cotton (glandless, recessive trait) was water-emasculated, the stigma allowed to dry, and the flower pollinated with pollen from 'Paymaster HS-26' cotton (glanded, dominant trait). Evaluation of 66 flowers revealed that 100% of the seedlings obtained from these crosses had the glanded phenotype, thereby showing that no self-pollination had occurred. In conclusion, this study demonstrates the use of water as an effective emasculation tool for hybrid cotton production that does not result in female sterility and maintains fruit retention and seed set following subsequent pollination with pollen from another flower.

Production of cotton (*Gossypium hirsutum* L.) hybrids by hand pollination is preceded by the removal of the anthers from the recipient flower. The emasculation of the recipient flower usually occurs the afternoon before they are pollinated. The major advantage of this method is that self-contamination is virtually eliminated; however, the high afternoon temperatures impair worker efficiency, usable pistillate flowers are missed, and protective covers may be blown off the stigmas, thus further reducing the number of pistillate flowers (Wilson and Strapp, 1984). Wilson and Strapp (1984) suggested that some breeders could overcome these potential problems by emasculating the flowers the morning of the day of pollination, but this introduced serious problems with the possibility of self-contamination. In addition to concerns about self-contamination, the process of flower emasculation raises concerns about injury to the flower and the possibility of cotton shedding a high percentage of flowers if the mechanical injury is too severe (Brown and Lee, 1976). Because of the concerns with existing cotton flower emasculation systems, an alternative method for emasculation is desirable. Several reports in the literature about environmental impacts on flower set and pollen viability have provided clues for developing a novel protocol for cotton flower emasculation.

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Cotton research in the 1920s, 1930s, and 1940s found that rain during the morning hours resulted in decreased boll set (Lloyd, 1920; Pearson, 1949). Pearson (1949) analyzed daily fluctuations in humidity, rainfall, and temperature from successive days of flowering in relation to flower set and mote formation. The ovules that failed to ripen into mature seeds developed into aborted structures that varied in degrees of seed, fiber, and embryo development and were defined as "motes" (Pearson, 1949). She concluded that rain during the time of pollination, even that falling shortly thereafter, affected the number of ovules receiving pollen tubes by interfering either with pollen deposition on the stigma or with pollen tube growth. Rains occurring on 5 Aug. 1937 and 3 Aug. 1939 came at the critical time and in sufficient amounts to impact boll set. For the first date, 13 bolls were set compared with 182 for 4 August and 233 for 6 August. For 3 Aug. 1939, 13 bolls were set compared with 80 on 2 August and 48 on 4 August (Pearson, 1949). Pearson (1949) stated that the amount of shedding for days on which heavy rains occurred during the forenoon would usually be so extensive that a large mote production for the relatively few bolls matured would have little influence on the total mote production of the entire crop. Clearly, these findings suggest an extreme sensitivity of cotton flowers to moisture at the time of pollination.

In a review of the physiological aspects of flower and fruit set in cotton, Stewart (1986) described the problems researchers faced when studying in vitro germination and tube growth of cotton pollen. He reported that studies of cotton pollen had been limited by the extreme sensitivity of cotton pollen to moisture. Whenever the grains or pollen tubes contacted available water, they ruptured. The result of the research was a myriad of nonaqueous, aqueous gels, or high-osmolarity solution protocols to obtain some degree of pollen germination and tube growth (Miravalle, 1965; Bronkers, 1961; Hancock, 1949; Vasil, 1958; Taylor, 1972; Wauford, 1979).

This paper describes a cotton flower emasculation protocol that capitalizes on the moisture sensitivity of cotton pollen and helps eliminate many of the concerns associated with the traditional emasculation procedures. The procedure occurs the day of pollination, does not mechanically injure the petals or corolla of the cotton flower, and produces normal seed set upon subsequent pollination of the emasculated flower.

MATERIALS AND METHODS

Plant Culture Protocols

Cotton 'Paymaster HS-26' seeds were the planted into hydroponic rock wool slabs (15 cm × 90 cm × 8 cm, w × l × d) that had been saturated with Peters Professional water-soluble fertilizer {0.95 g L^{-1} 5–11–26 HYDRO-SOL (Scotts-Sierra Hortic. Products Co., Maryville, OH), supplemented

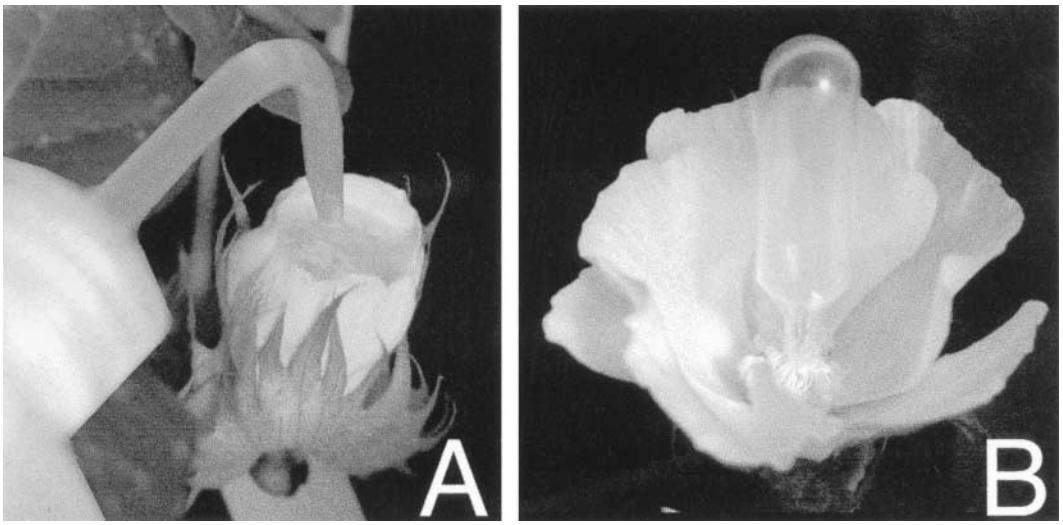

Fig. 1. Photographs of the water treatment of cotton flowers showing (A) the use of a water bottle to fill an opening flower with water and (B) the covering of the stigma with a plastic bulb following the water treatment.

with 0.475 g L^{-1} calcium nitrate [$Ca(NO_3)_2$; Ca Hydro Agri North America, Tampa, FL] and 0.238 g L^{-1} magnesium sulfate ($MgSO_4$; Scotts-Sierra Hortic. Products Co., Maryville, OH)}. Three seeds were planted per pad, and a total of 20 hydroponic rock wool slabs were placed on benches in a greenhouse. Nutrients were maintained using a nonrecycling hydroponic watering system. Plants were grown under greenhouse conditions (28 ± 5°C air temperature) for 120 d under natural-light conditions. The evaluation of flowers from 60 plants was replicated three times over a 12-mo period.

Water Treatment of Flowers

The effect of water treatments on pollen–stigma interactions was evaluated at different times throughout the day. The petals of opened flowers were cupped with one hand, and water was applied with a squirt bottle using the other hand (Fig. 1A). Once the flower was filled with water, hand cupping ceased. In initial studies, the water treatments occurred when the petals began to open, forming a natural cup-shaped flower that would hold water. The captured water was allowed to interact with the flower components for a range of incubation times from 30 s to 30 min. Following the water treatments, water remaining in the flower was poured off, and the stigmas were covered with a plastic dropper bulb to prevent inadvertent pollination (Fig. 1B). Treated flowers were then monitored to determine if the water treatments effected boll set, seed production, or both. A minimum of 10 flowers were evaluated per treatment. After identifying the rapidity of the water destruction of the pollen in the initial experiments, flowers were only cupped in the hand during the application of the water in subsequent water treatments, allowing the water to run out as the residual water clinging to the anthers was sufficient to destroy existing pollen.

Callose Staining to Monitor Pollen Tube Growth

Cotton pistils were prepared for visualization of pollen tube growth according to a procedure adapted from Vogt et al. (1994). Control and water-treated flowers were harvested, and bracts, corollas, and stamens were removed. Pistils were fixed in ethanol–acetic acid mixture (3:1 v/v) for 24 h. The tissue was rinsed with 1 *M* potassium phosphate (K_2HPO_4) buffer, pH 7.0, and incubated in 1 *M* NaOH for 3 to 24 h to clear the tissue. Pistils were stained with 0.005% (w/v) decolorized analine blue in 0.1 *M* K_2HPO_4, pH 9.0, for 2 to 4 h; infiltrated with 100% (w/v) glycerol for 1 h; and mounted on glass slides. Pistils and pollen tubes were visualized with a fluorescence microscope (Olympus BX60, Olympus America, Melville, NY) (emission l = 410 nm), and video images from a MTI 3 CCD camera (PAGE-MTI, Michigan City, IN) were captured and saved as TIFF images. A minimum of five flowers per treatment were evaluated.

Water Treatment of Dehisced Pollen

Cotton flowers were harvested between 0900 and 1000 h CST, and pollen was collected by inverting the flower and tapping to shake loose pollen that had dehisced. Collected pollen was placed on a glass microscope slide and photographed before adding water. A drop of water was added to the pollen, which was incubated for 2 min and photographed again to determine any direct structural changes that might arise from the water treatment.

Water Treatment Effects on Boll Set and Seed Production

To determine if the effectiveness of the water emasculation changed throughout the day, 20 cotton flowers were treated with enough water to cover the anthers, approximately 5 to 10 mL of water for 1 min; then, excess water was poured out of the flower, and a plastic bulb was placed over the stigma at 0800 h. The procedure was repeated hourly on different flowers until 1600 h. No additional pollen was added to the flowers so that the effect of water on existing pollen could be evaluated. Water-treated flowers were monitored for boll set and seed production, and the average number of seeds per boll were identified and compared with naturally pollinated flowers (control). Means and standard errors were calculated.

Evaluation of Stigma–Pollen Functionality following a Water Treatment

To evaluate the effectiveness of flower pollination following a water pretreatment, flower petals were cupped and the flower filled with water for 30 s at 0830, 10:30, 1230, and 1430 h. Immediately following the water treatment, the stigmas were covered with a plastic dropper bulb until pollinated. Immediately before pollination, the covers were removed from the stigmas, and the stigmas were blotted dry with a Kimwipe (Kimberly-Clark Corp., Rosewell, GA). One-half of the water-treated

flowers from each emasculation time was pollinated at 1435 h with pollen from untreated flowers. Flowers were then monitored to determine if the timing of the water treatments affected boll set or seed production. Seed numbers per boll from the treated flowers were compared with the number of seeds per boll in naturally pollinated flowers. Five flowers were evaluated per treatment at each time point. Means and standard errors are provided in the data presentation.

Evaluation of the Effectiveness of Water Emasculation

Cotton Paymaster HS-26 and 'Gregg 65' seeds were planted into hydroponic rock wool slabs [15 by 90 by 8 cm (width by length by depth)] that had been saturated with Peters Professional water-soluble fertilizer [0.95 g L^{-1} 5-11-26 HYDROSOL (Scotts-Sierra Hortic. Products Co., Maryville, OH), supplemented with 0.475 g L^{-1} calcium nitrate (Ca Hydro Agri North America, Tampa, FL) and 0.238 g L^{-1} magnesium sulfate (Scotts-Sierra Hortic. Products Co., Maryville, OH)]. Three seeds were planted per pad, a total of 20 hydroponic rock wool slabs were placed on benches in a greenhouse, and nutrients were maintained at optimal levels using a nonrecycling hydroponic watering system. Plants were grown under greenhouse conditions (28 ± 5°C air temperature) for 120 d under natural light conditions. Over a 1-wk period, a total of 66 flowers from 12 different Gregg 65 plants were filled with water for 1 min between 0900 and 1300 h. Excess water was poured out of the flower, and a plastic bulb was placed over the stigma to prevent inadvertent pollination. Pollen collected from the glanded Paymaster HS-26 cotton was harvested and used to pollinate the water-emasculated glandless Gregg 65 flowers within 30 min of water emasculation. Following boll maturation, seeds were harvested, and the ginned and delinted seeds were planted into the hydroponic rock wool slabs. Seedlings were evaluated for the presence or absence of gossypol glands 2 wk after planting. If the water emasculation was effective in destroying the Gregg 65 pollen, then all seedlings should contain the dominant-marker gossypol glands. If the water emasculation failed to destroy the existing pollen, then some seedlings should be glandless.

RESULTS

The effectiveness of water as an emasculating agent for cotton flowers on the day of pollination was evaluated at the morphological and functional levels. Water applied to the opening flower (Fig. 1A) was found to reduce the number of pollen grains associated with the stigma (Fig. 2B) and inhibit pollen tube production in the grains that remained (Fig. 2B and 3B). Fluorescence microscopy of pollen tube growth on pistils of a control and water-treated flower are shown in the composite photographs of Fig. 2. Control flowers showed large numbers of pollen grains attached to the sides of the stigma (Fig. 2A), and pollen tubes arising from the pollen grain appeared as white lines moving horizontally toward the stigma's transport tissues (Fig. 2A and 3A). By comparison, few pollen grains were associated with the stigma from the water-treated flower (Fig. 2B). The pollen tubes so visible in the control flowers were not apparent in the water-treated samples (Fig. 2B and 3B). There was a noticeable change in the appearance of the papillae of the water-treated flower (Fig. 2B). Many of the papillae along the periphery of the photomicrograph appear highly fluorescent following the water treatment. At first glance, one might mistake the modified papillae as pollen tubes; however, upon closer examination (Fig. 2B and 3B), it is clear that there are no pollen tubes associated with the water-treated pollen.

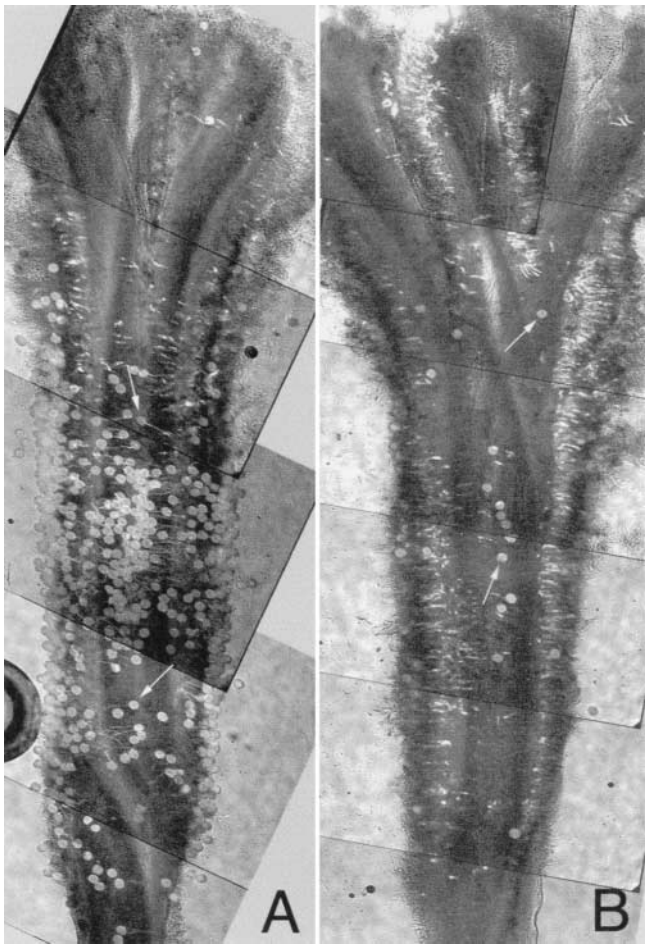

Fig. 2. Fluorescence micrographs showing the extent of pollen germination and tube growth on (A) control and (B) water-treated pistils. Arrows are provided to highlight representative pollen grains (A) with and (B) without pollen tube formation.

The absence of pollen tubes on the stigmas of the water-treated flowers may be a result of a direct effect of the water on the pollen, the stigma, or both. To further evaluate the effects of the water treatment on this process, isolated pollen was evaluated for possible morphological and physiological changes. A photomicrograph of dehisced cotton pollen before (Fig. 4A) and 2 min after (Fig. 4B) water treatment help to provide insights as to the reason for the lack of pollen tubes on the pollen grains associated with the water-treated flowers. The addition of water to the isolated cotton pollen resulted in the rupturing of the pollen grain and the extrusion of the cytoplasm into the surrounding medium (Fig. 4B). The time course for pollen grain rupturing ranges from a few seconds to several minutes. Isolated pollen that had been water-treated for 30 min was used to pollinate 10 water-treated flowers, and flower drop and seed set were compared with control pollen that had not been water-treated and used to pollinate water-treated flowers. The results showed that all 10 flowers pollinated with water-treated pollen dropped from the plants and failed to set any seeds while 10

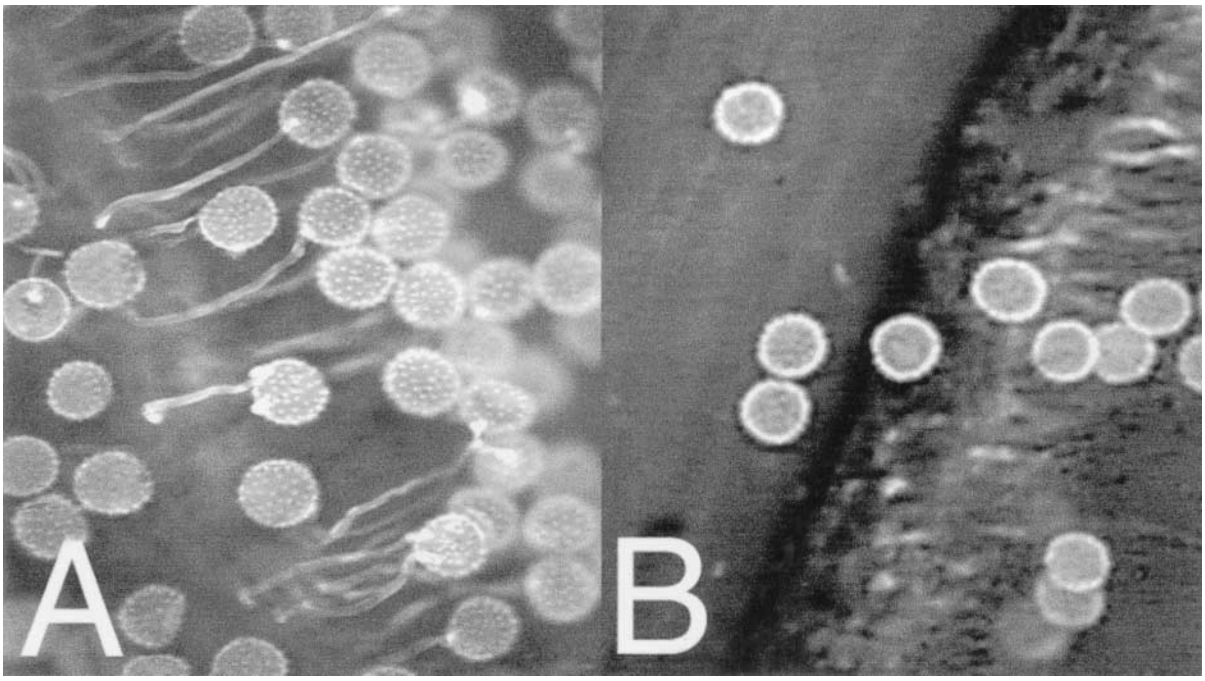

Fig. 3. Fluorescence micrographs showing the extent of pollen germination and tube growth on (A) control and (B) water-treated pistils. Pollen tube growth into the transport tissue of the pistil is shown in A while B shows that no pollen tube development was observed from the pollen grains of the water-treated flowers.

flowers treated with control pollen resulted in normal boll set and seed production.

The effect of water treatment on isolated pollen grains suggested that adding water to the cotton flowers would be an effective emasculation protocol. These data, however, did not provide any indication of the timing of the water treatment on the efficiency of flower emasculation. Flowers were treated with water at 0800, 0900, 1000, 1100, 1200, 1300, 1400, 1500, and 1600 h and evaluated for the efficiency of the sterilization procedure. The time course of water emasculation of cotton flowers is shown in Fig. 5. Water emasculation of the flower occurred at all times evaluated. The efficiency of the emasculation process tracked the timing of the dehiscing of the pollen within the flower. The most efficient emasculation occurred between 0900 and 1300 h. The few *seeds* that remained following the water emasculation were in fact motes, and no viable seed was obtained based on germination tests in the rock wool pads.

The effect of water as an emasculation tool is evident

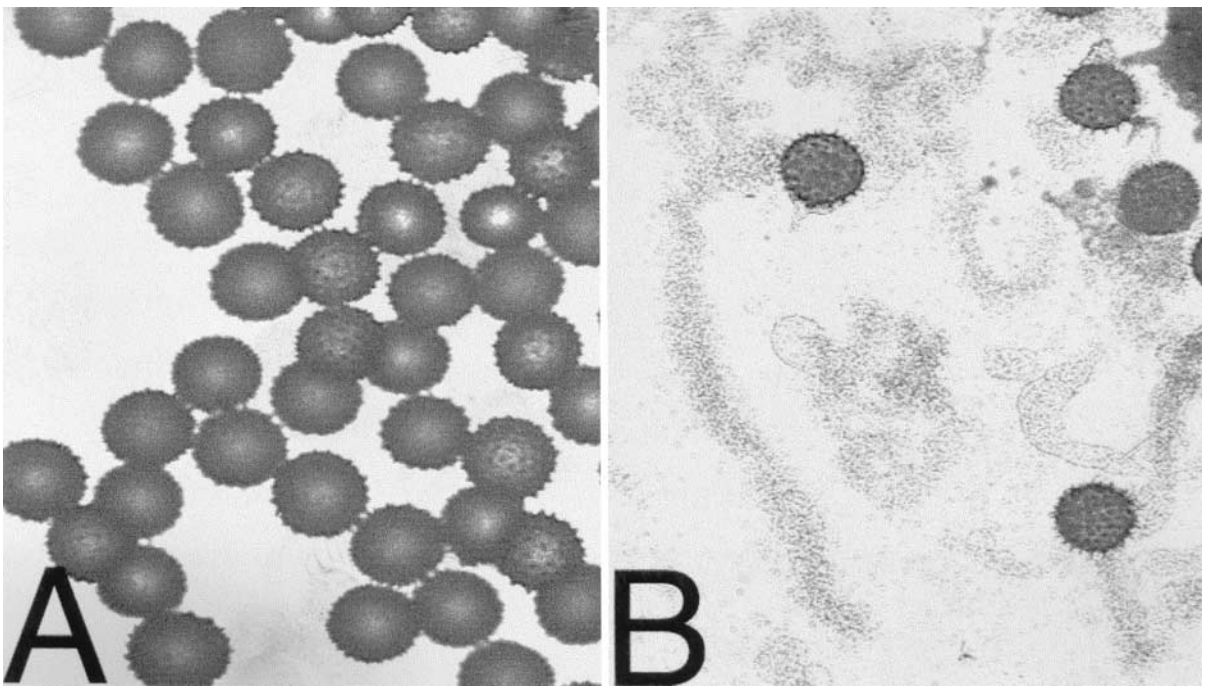

Fig. 4. Photomicrographs of cotton pollen (A) before and (B) after water treatment. Cotton pollen grains burst upon exposure to water, and the cytoplasmic contents stream out of the pollen grain into the surrounding water (B).

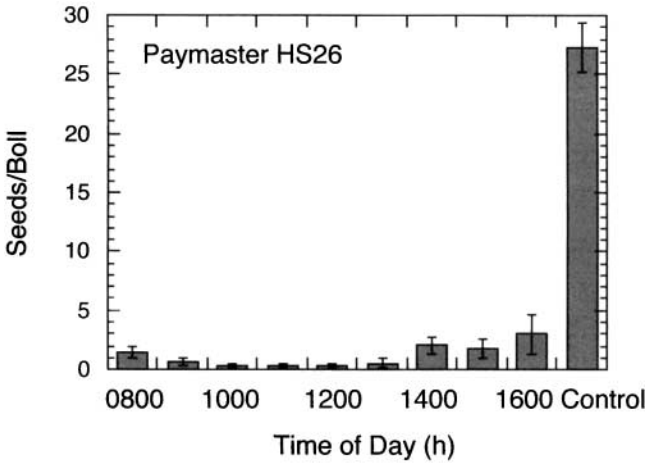

Fig. 5. Determination of the average number of seeds per boll observed for flowers treated with water at different times during the day. Treated flowers are compared with self-pollinated flowers (control). An average of 20 flowers were evaluated at each time point for the Paymaster HS26 variety. Means and standard errors are provided.

from the data presented in this study. The pollen data, however, did not rule out possible inhibitory effects of water on the stigma of the flower. Studies were conducted to evaluate the receptivity of water-treated stigmas to viable cotton pollen. The results of the time course of water treatment showed that water-treated flowers were receptive to viable pollen and that the number of seeds per boll in the hand-pollinated flowers was similar to that of self-fertilized controls (Fig. 6). There was an effect of the sterilization timing on subsequent seed set, as evidenced by the reduction in the number of seeds per boll in the 1430-h treatment. We cannot rule out whether the reduced seed set at 1430 h was the result of more moisture being present on the stigma of these flowers because they had just been treated with water before pollination, or if there is a reduced seed set because the flower may be undergoing the senescence process that occurs in all cotton flowers

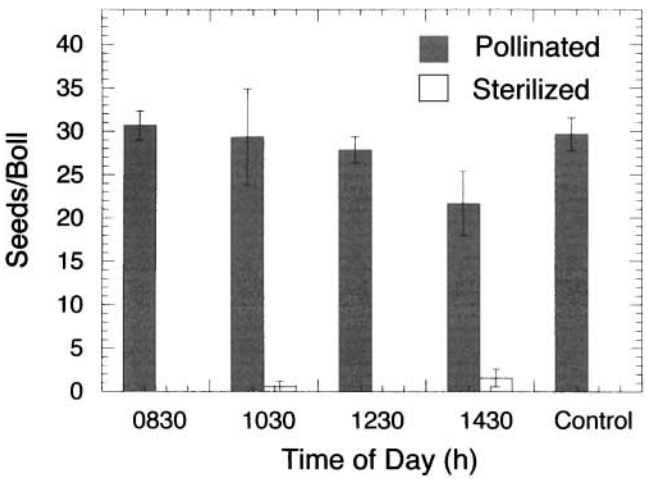

Fig. 6. Evaluation of the effect of the water treatment timing on stigma receptiveness to pollen. One-half of the water-treated flowers were pollinated at 1435 h, and the average number of seeds per boll were determined at boll maturity. Five flowers were evaluated per treatment at each time point. Means and standard errors are provided.

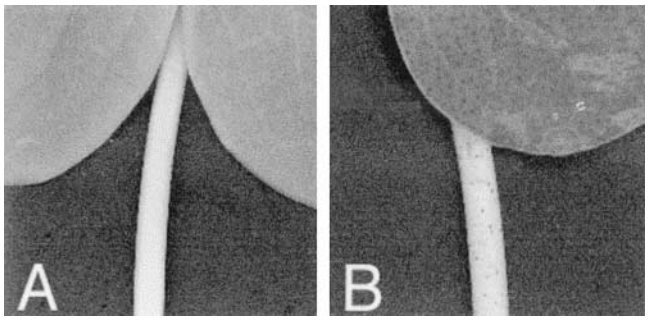

Fig. 7. Photographs of (A) Gregg 65 and (B) Paymaster HS-26 seedlings demonstrating the (A) absence and (B) presence of gossypol glands on the hypocotyls and cotyledons.

at the end of the day. The absence or reduction in seed set in the water-treated flowers that were not pollinated (open bars) again shows the effectiveness of the water emasculation procedure in preventing seed set (Fig. 6).

To evaluate further the effectiveness of the water emasculation protocol, flowers of Gregg 65 cotton, a glandless (recessive trait) variety (Fig. 7A), were water-emasculated and subsequently hand-pollinated with pollen from Paymaster HS-26 cotton, a glanded (dominant trait) variety (Fig. 7B). All of the 66 flowers set seed, averaging 27 seeds per boll. If self pollination occurred, then some of the seedlings derived from these crosses would be glandless. Following germination, seedlings were evaluated for gossypol glands on the hypocotyls. All of the seedlings evaluated carried the dominant glanded trait. No glandless seedlings were obtained from the 66 individual water emasculations. These results demonstrate the effectiveness of the water in disrupting existing pollen in the flower.

DISCUSSION

Production of hybrid cotton in breeding programs requires the attainment of male sterility without substantial female sterility or loss of reproductive vigor. Numerous methods have been employed, including the use of genetic male sterility, chemosterilents, or emasculation (Brown and Lee, 1976; Olvey et al., 1981; Olvey, 1992; Ray and Longoria, 1986). To date, emasculation remains the most widely used and reliable method to remove male gametes in breeding programs. Traditional methods for cotton flower emasculation involve the removal of the anthers in the late bud stage and can result in varying degrees of tissue damage. Tissue damage associated with mechanical emasculation has been shown to enhance fruit shed in many cotton varieties and wild accessions (Brown and Lee, 1976). Studies have evaluated the addition of hormones to the androecium at emasculation and shown that they can help reduce fruit loss (Brown and Lee, 1976). Wilson and Strapp (1984) evaluated the feasibility of emasculating cotton flowers the morning of the day of pollination rather than at the traditional time of the afternoon before pollination. They reported that hand-emasculating flowers even as early as 0200 h on the day of pollination caused some anthers to burst and self-contaminate the stigmas. To overcome possible self-contamination, Wilson and Strapp (1984) began treating mechanically emasculated flowers with 30% ethanol, according to the

method described by Lee (1980), to kill and wash off unwanted pollen. They found that the washing of mechanically emasculated flowers allowed them to take advantage of morning emasculations.

New emasculation procedures are desirable because of tissue damage associated with mechanical emasculation and because of the inherent problems associated with finding and emasculating cotton flowers the day before pollination. The research reported in this study investigated the potential use of water to emasculate cotton flowers the day of pollination. Water was chosen as a potential emasculation tool because of Pearson's (1949) report of rain resulting in cotton fruit loss and because studies developing media for cotton pollen germination in vitro went to great lengths to avoid rupturing pollen grains or tubes because of low osmotic solutions (Stewart, 1986). The results of this study show that cotton pollen is very sensitive to water and that addition of water to cotton flowers during the midmorning or afternoon hours results in pollen destruction. The emasculation of cotton flowers with water destroyed the pollen yet left the stigma receptive to the addition of pollen used in making crosses. The flower petals showed no signs of injury, opening normally during the morning hours and senescing at the end of the day in the same manner as control flowers.

There are numerous advantages of the water emasculation system described in this paper compared with mechanical emasculation procedures. Because this procedure is performed the day of pollination, one avoids the afternoon temperature extremes that would impair worker efficiency. Another advantage of the water emasculation procedure is that opening flowers are more easily detected than cotton flowers in the *candle* stage, thereby ensuring a better coverage of the daily flower production. The morning water emasculation reduces the time that protective covers are present before pollination, thereby limiting the time during which they might be blown off. Finally, the protocol is rapid and technically simple. The treatment destroys only the pollen and leaves the stigma receptive to pollen used for crosses. The only concern required for emasculation is that the water treatment saturates all of the anthers and pollen grains within the flower, thereby eliminating the possibility of self-pollination. When hand-pollinating following water emasculation, it is essential that the receiving stigma be free of water before pollination. If water droplets remain on the stigma, the added pollen will rupture, and seed set will be reduced. Water-treated stigmas that were not covered with the plastic bulbs evaporated surface moisture within 15 min. The presence of the plastic bulb maintained a high moisture level around the stigma until removed. Practical application of this information in a field or greenhouse breeding program would require entering the field or greenhouse after the flowers have dehisced, filling the cupped flower with water, and then letting the excess water pour out. After emasculating 5 to 10 flowers, return to the first flower, dry the stigma surface with a towel or Kimwipe, and pollinate with pollen from another flower. The total time required per flower is approximately 1 min.

In summary, this study showed that water could be used to efficiently emasculate cotton flowers the day of pollination. The pollen was destroyed by the water apparently because of large osmotic differences between the water and the pollen cytoplasm. Morphological analysis of isolated pollen grains before and after water treatment showed the exudation of pollen cytoplasm into the surrounding water medium within seconds of the water treatment. The water treatment resulted in the loss of fruiting bodies unless the flowers were subsequently pollinated with viable pollen. The bolls that were set following hand pollination of water-emasculated flowers produced seed numbers equivalent to self-pollinated controls.

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